

SEROEPIDEMIOLOGY OF *TOXOPLASMA GONDII* IN ZOO ANIMALS IN SELECTED ZOOS IN THE MIDWESTERN UNITED STATES

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ABSTRACT: *Toxoplasma gondii* infections in zoo animals are of interest because many captive animals die of clinical toxoplasmosis and because of the potential risk of exposure of children and elderly to *T. gondii* oocysts excreted by cats in the zoos. Seroprevalence of *T. gondii* antibodies in wild zoo felids, highly susceptible zoo species, and feral cats from 8 zoos of the midwestern United States was determined by using the modified agglutination test (MAT). A titer of 1:25 was considered indicative of *T. gondii* exposure. Among wild felids, antibodies to *T. gondii* were found in 6 (27.3%) of 22 cheetahs (*Acinonyx jubatus jubatus*), 2 of 4 African lynx (*Caracal caracal*), 1 of 7 clouded leopards (*Neofelis nebulosa*), 1 of 5 Pallas cats (*Otocolobus manul*), 12 (54.5%) of 22 African lions (*Panthera leo*), 1 of 1 jaguar (*Panthera onca*), 1 of 1 Amur leopard (*Panthera pardus orientalis*), 1 of 1 Persian leopard (*Panthera pardus saxicolor*), 5 (27.8%) of 18 Amur tigers (*Panthera tigris altaica*), 1 of 4 fishing cats (*Prionailurus viverrinus*), 3 of 6 pumas (*Puma concolor*), 2 of 2 Texas pumas (*Puma concolor stanleyana*), and 5 (35.7%) of 14 snow leopards (*Uncia uncia*). Antibodies were found in 10 of 34 feral domestic cats (*Felis domesticus*) trapped in 3 zoos. *Toxoplasma gondii* oocysts were not found in any of the 78 fecal samples from wild and domestic cats. Among the macropods, antibodies were detected in 1 of 3 Dama wallabies (*Macropus eugenii*), 1 of 1 western grey kangaroo (*Macropus fuliginosus*), 1 of 2 wallaroos (*Macropus robustus*), 6 of 8 Bennett's wallabies (*Macropus rufogriseus*), 21 (61.8%) of 34 red kangaroos (*Macropus rufus*), and 1 of 1 dusky pademelon (*Thylogale brunii*). Among prosimians, antibodies were detected in 1 of 3 blue-eyed black lemurs (*Eulemur macaco flavifrons*), 1 of 21 ring-tailed lemurs (*Lemur catta*), 2 of 9 red-ruffed lemurs (*Varecia variegata rubra*), and 2 of 4 black- and white-ruffed lemurs (*Varecia variegata variegata*). Among the avian species tested, 2 of 3 bald eagles (*Haliaeetus leucocephalus*) were seropositive. Among 7 possible risk factors, sex, freezing meat temperature (above -13°C vs. below -13°C), washing vegetables thoroughly, frequency of feral cat sightings on zoo grounds (occasionally vs. frequently), frequency of feral cat control programs, capability of feral cats to enter hay/grain barn, and type of animal exhibit, exhibiting animals in open enclosures was the only factor identified as a significant risk (OR 3.22, $P = 0.00$).

Toxoplasma gondii infections are widely prevalent in human beings and other animals worldwide (Dubey and Beattie, 1988). Although infections in most animals and humans are asymptomatic, toxoplasmosis can cause severe illness in congenitally infected children and those with depressed immunity. Certain species of animals (Australasian marsupials, New World monkeys, lemurs, among others) can die of severe toxoplasmosis. *Toxoplasma gondii* infections in zoo animals are of particular interest because many species of animals (Pallas cats, canaries, finches, marsupials) in captivity die of clinical toxoplasmosis, and there is potential risk of exposure of children and elderly to *T. gondii* oocysts excreted by cats in the zoos.

The purpose of the present study was to determine the seroprevalence of *T. gondii* antibodies in wild zoo felids, highly susceptible zoo species, and feral domestic cats from 8 zoos of the Midwestern United States.

MATERIALS AND METHODS

Participating zoos and sample collection

A total of 269 serum samples was collected from 8 zoos in the midwestern United States (Toledo Zoo, Toledo, Ohio; Columbus Zoo and Aquarium, Columbus, Ohio; Cleveland Metroparks Zoo, Cleveland, Ohio; Cincinnati Zoo and Botanical Garden, Cincinnati, Ohio; Indianapolis Zoo, Indianapolis, Indiana; Milwaukee County Zoo, Milwaukee, Wisconsin; St. Louis Zoo, St. Louis, Missouri; and Louisville Zoo, Louisville, Kentucky). Serum or plasma samples (0.2–1 ml) were opportunistically collected from banked specimens from 1 January 2003 through 1 June 2005. If available, a second and a third sample from the same animal, collected 6–12 mo later, was also obtained. Results of the last bleeding were used to determine seropositivity. Samples were

either picked up directly by the senior author or shipped overnight in cold packs to The Ohio State University (OSU) where they were stored at -80°C until shipped to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, Maryland, for serological evaluation. Data on age, sex, and health status on each animal sampled was collected from the zoo records. The animals included species from wild felids, marsupials, prosimians, New World monkeys, and specific species of birds (passeriformes, columbiformes, and birds of prey).

Collection of serum samples from feral cats

Feral cats trapped within the grounds of zoo B, D, and H were captured using humane methods and anesthetized, and blood samples were collected.

Collection of felines feces

Sixty-six (58 from individual animals and 8 pooled samples from cagemates) were collected from nondomestic felids in all the participating zoos. This was a cross-sectional study, and samples were collected at 1 time point fresh from the floor of the captive cats' enclosures during routine cleaning practices and from the cages. Twelve fecal samples were collected directly from the rectum of feral cats. Fecal samples were placed in plastic containers, labeled, and stored at 4°C for no more than 7 days before shipment to the APDL, Beltsville, Maryland, for *T. gondii* examination. Feces (5 g) were floated in sugar solution (sp. gr. 1.18), examined microscopically for oocysts, and mixed with 2% sulfuric acid, then aerated on a shaker for 1 wk. After neutralization, the samples were bioassayed in mice as described (Dubey et al., 2005).

Epidemiological investigation

Seven risk factors—sex, freezing meat temperature (above -13°C vs. below -13°C), washing vegetables thoroughly, frequency of feral cat sightings on zoo grounds (occasionally vs. frequently), frequency of feral cat control programs, capability of feral cats to enter hay/grain barn, and type of animal exhibit (open vs. closed exhibits)—were evaluated using univariate logistic regression analysis.

Serological examination

Sera of animals were tested for *T. gondii* antibodies with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987). Titers of 1:25 or higher were considered indicative of *T. gondii* exposure.

Received 4 September 2007; revised 18 October 2007; accepted 19 October 2007.

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TABLE I. Summary of zoo animals seropositive to *T. gondii*.

Species	No. of seropositive animals								No. positive/no. tested	
	Zoo A	Zoo B	Zoo C	Zoo D	Zoo E	Zoo F	Zoo G	Zoo H	% Positive	
Felids										
Cheetah (<i>Acynonyx jubatus jubatus</i>)	1	1	1	0	0	0	1	2	6/22	27.3
Lynx (<i>Caracal caracal</i>)	0	0	0	0	1	1	0	0	2/4	50.0
Clouded leopard (<i>Neofelis nebulosa</i>)	0	1	0	0	0	0	0	0	1/7	14.3
Pallas cat (<i>Otocolobus manul</i>)	1	0	0	0	0	0	0	0	1/5	20.0
African lion (<i>Panthera leo</i>)	0	0	4	4	2	0	0	2	12/22	54.5
Jaguar (<i>Panthera onca</i>)	0	0	0	0	1	0	0	0	1/1	100
Amur leopard (<i>Panthera pardus orientalis</i>)	0	0	0	0	0	0	1	0	1/1	100
Persian leopard (<i>Panthera pardus saxicolor</i>)	0	1	0	0	0	0	0	0	1/1	100
Amur tiger (<i>Panthera tigris altaica</i>)	0	0	0	3	1	1	0	0	5/18	27.8
Fishing cat (<i>Prionailurus viverrinus</i>)	0	0	0	0	1	0	0	0	1/4	25.0
Puma (<i>Puma concolor</i>)	0	0	2	0	1	0	0	0	3/6	50.0
Texas puma (<i>Puma concolor stanleyana</i>)	0	0	0	0	0	2	0	0	2/2	100
Snow leopard (<i>Uncia uncia</i>)	0	0	0	0	2	1	2	0	5/14	35.7
Domestic cat (<i>Felis domesticus</i>)	0	6	0	2	0	0	0	2	10/34	29.4
Macropods										
Dama wallaby (<i>Macropus eugenii</i>)	0	1	0	0	0	0	0	0	1/3	33.3
Western gray kangaroo (<i>Macropus fuliginosus</i>)	0	1	0	0	0	0	0	0	1/1	100
Wallaroo (<i>Macropus robustus</i>)	0	0	0	0	1	0	0	0	1/2	50.0
Wallaby (<i>Macropus rufogriseus</i>)	0	3	1	0	0	0	0	2	6/8	75.0
Red kangaroo (<i>Macropus rufus</i>)	0	4	1	11	0	5	0	0	21/34	61.8
Dusky pademelon (<i>Thylogale brunii</i>)	0	1	0	0	0	0	0	0	1/1	100
Prosimians										
Blue-eyed black lemur (<i>Eulemur macaco flavifrons</i>)	0	0	0	1	0	0	0	0	1/3	33.3
Ring-tailed lemur (<i>Lemur catta</i>)	0	0	0	1	0	0	0	0	1/21	4.8
Red-ruffed lemur (<i>Varecia variegata rubra</i>)	0	0	0	2	0	0	0	0	2/9	22.2
Black/white-ruffed lemur (<i>Varecia variegata variegata</i>)	2	0	0	0	0	0	0	0	2/4	50.0

Statistical analysis

The relationship between the outcome (*T. gondii* seropositivity) and the independent variables was modeled using logistic regression because of the nature of the outcome variable following the procedure described by Hosmer and Lemeshow (2000). Odds ratios and 95% confidence intervals were calculated to determine if there were significant differences among the variables. A value of $P \leq 0.05$ was considered significant. Data obtained from the epidemiological survey related to management practices among zoos and *T. gondii* prevalence rates in the zoo collection were evaluated by univariate logistic regression.

To determine if *T. gondii* prevalence in zoos was independent to the prevalence in the existing feral cat population, Spearman's Rank test and Pearson X^2 were run for each of the institutions that submitted feral cat serum samples. All statistical analysis was performed using Stata Statistical Software, Release 7.0 (Stata Corporation, College Station, Texas).

RESULTS

Serological prevalence in zoo animals

Antibodies to *T. gondii* were found in felids, macropods, and prosimians, and data from each zoo are given in Table I and Table II. Among the avian species tested, 2 of 3 bald eagles (*Haliaeetus leucocephalus*) were seropositive with MAT titers of 1:25. Antibodies to *T. gondii* were not detected in 6 Rüppell's vultures (*Gyps rueppellii*), in 2 each of Cinereous vultures (*Aegypius monachus*), red-tailed hawks (*Buteo jamaicensis*), turkey vultures (*Cathartes aura*), barred owls (*Strix varia*), lap-pet-faced vultures (*Torgos tracheliotus*), Bali mynahs (*Leucop-*

sar rothschildi), and 1 each of black vulture (*Coragyps atratus*), peregrine falcon (*Falco peregrinus*), Victoria crowned pigeon (*Goura victoria*), spectacled owl (*Pulsatrix perspicillata*), king vulture (*Sarcorhamphus papa*), and barn owl (*Tyto alba*). Most (88%) birds sampled were birds of prey.

Antibodies to *T. gondii* were not detected in any of the 36 New World monkeys, including 1 black howler monkey (*Alouatta caraya*), 4 black-handed spider monkeys (*Ateles geoffroyi*), 5 Reed titis (*Callicebus moloch donacophilus*), 3 Goeldi's monkeys (*Callimico goeldii*), 1 black-tufted ear marmoset (*Callithrix kuhlii*), 4 woolly monkeys (*Lagothrix lagothricha*), 1 golded-headed lion tamarin (*Leontopithecus chrysomelas*), 3 golden lion tamarins (*Leontopithecus rosalia*), 8 pale headed sakis (*Pitheca pitheca*), and 6 Geoffroy's tamarins (*Saguinus geoffroyi*).

All fecal samples from felids were negative for *T. gondii* oocysts. Feral cats were observed more frequently in zoos A, B, C, E, and H than in zoos F and G. Most (70.6%) of the feral cats trapped were adults, and all seropositive cats were adults. Neither frequency of feral cat control nor having a cat-proof hay barn was identified as a risk factor for *T. gondii* infection in zoo animals. Nevertheless, zoos that control feral cats based on population density are 15% more likely to have seropositive animals than those institutions that have a weekly active feral cat control program ($P = 0.61$). Furthermore, zoos where cats were able to enter the hay barn were 50% more likely than zoos

TABLE II. Summary of zoo animals seropositive to *T. gondii* with respective MAT titers.

Species	No. positive/no. tested	MAT titers of seropositive animals							
		1:25	1:50	1:100	1:200	1:400	1:800	1:1,600	≥1:3,200
Felids									
Cheetah (<i>Acynonyx jubatus jubatus</i>)	6/22	0	1	0	1	0	2	1	1
Lynx (<i>Caracal caracal</i>)	2/4	0	0	0	0	1	0	0	1
Clouded leopard (<i>Neofelis nebulosa</i>)	1/7	0	0	0	0	0	1	0	0
Pallas cat (<i>Otocolobus manul</i>)	1/5	0	0	0	0	0	0	0	1
African lion (<i>Panthera leo</i>)	12/22	0	0	1	1	5	0	3	2
Jaguar (<i>Panthera onca</i>)	1/1	0	1	0	0	0	0	0	0
Amur leopard (<i>Panthera pardus orientalis</i>)	1/1	0	0	1	0	0	0	0	0
Persian leopard (<i>Panthera pardus saxicolor</i>)	1/1	0	0	0	0	0	0	0	1
Amur tiger (<i>Panthera tigris altaica</i>)	5/18	0	1	1	0	1	2	0	0
Fishing cat (<i>Prionailurus viverrinus</i>)	1/4	1	0	0	0	0	0	0	0
Puma (<i>Puma concolor</i>)	3/6	0	1	1	1	0	0	0	0
Texas puma (<i>Puma concolor stanleyana</i>)	2/2	0	0	0	1	1	0	0	0
Snow leopard (<i>Uncia uncia</i>)	5/14	0	0	0	1	1	1	1	1
Domestic cat (<i>Felis domesticus</i>)	10/34	1	2	2	0	1	0	2	2
Macropods									
Dama wallaby (<i>Macropus eugenii</i>)	1/3	0	0	0	0	0	0	0	1
Western gray kangaroo (<i>Macropus fuliginosus</i>)	1/1	0	0	0	0	0	0	0	1
Wallaroo (<i>Macropus robustus</i>)	1/2	0	0	0	0	0	0	0	1
Wallaby (<i>Macropus rufogriseus</i>)	6/8	1	0	0	0	1	0	0	4
Red kangaroo (<i>Macropus rufus</i>)	21/34	3	0	0	0	0	0	0	18
Dusky pademelon (<i>Thylogale brunii</i>)	1/1	0	0	0	0	0	0	0	1
Prosimians									
Blue-eyed black lemur (<i>Eulemur macaco flavifrons</i>)	1/3	0	0	0	0	0	0	0	1
Ring-tailed lemur (<i>Lemur catta</i>)	1/21	0	0	1	0	0	0	0	0
Red-ruffed lemur (<i>Varecia variegata rubra</i>)	2/9	0	0	0	0	0	0	0	2
Black/white-ruffed lemur (<i>Varecia variegata variegata</i>)	2/4	0	0	0	0	0	0	0	2

with cat-proof hay barns to have *T. gondii*-positive animals ($P = 0.14$). Univariate regression analysis demonstrated that animals that are kept at open exhibits were 3.22 times more likely to be positive to the parasite than those held in closed exhibits ($P = 0.00$).

In general, all participating zoos had similar food handling practices. Meat was frozen prior to offering it to the animals. Then again, freezing temperatures varied greatly between zoos, ranging from -2 to -20 °C. Surprisingly, zoos that maintained freezing temperatures below -13 °C were 37% more likely to have infected animals than zoos that preserve meat above this temperature; nonetheless this result was not statistically significant ($P = 0.27$). Although also not statistically significant, zoos that washed vegetables thoroughly before offering them to the animals were 90% less likely to have positive *T. gondii* animals than zoos that do not follow this practice ($P = 0.75$).

DISCUSSION

Serological prevalence in macropods

Among the Australasian marsupials, clinical toxoplasmosis is more severe in macropods, especially wallabies (Canfield et al., 1990). There are many reports of severe toxoplasmosis in macropods in zoos worldwide, including from the United States (Boorman et al., 1977; Dubey, Ott-Joslin et al., 1988; Miller et al., 1992; Adkesson et al., 2007). In the present study, all of

the marsupials tested were macropods, with 49% having titers of $\geq 1:3,200$. From this group, half of the animals were reported clinically unhealthy at the time of the serum collection. It is likely that many of these unhealthy individuals were suffering an active *T. gondii* infection at the time the sample was collected. In July 2004 there was an epizootic of toxoplasmosis in zoo D, resulting in the clinical death of 3 of 4 red kangaroos. Sera from 2003 and 2004 were submitted for the study. All 4 animals had negative titers ($\leq 1:25$) in their 2003 samples and had seroconverted with titers of $\geq 1:3,200$ in the 2004 samples.

Serological prevalence in wild felids

Clinical toxoplasmosis and oocyst shedding has been reported in several of the 13 species of the wild cats found to have *T. gondii* antibodies in the present study (reviewed in Dubey and Beattie, 1988; Silva et al., 2001). Of the wild felids, toxoplasmosis in the Pallas cat (*Otocolobus manul*) is of great significance. The natural habitat for Pallas cats is the high mountains in Tibet, western Siberia, Turkestan, and Mongolia. There are only a few Pallas cats in the zoos in the United States because of high mortality in newborns due to toxoplasmosis (Riemann et al., 1974; Dubey, Gendron-Fitzpatrick et al., 1988; Swanson, 1999; Kenny et al., 2002). Additionally, Pallas cats can shed *T. gondii* oocysts, further contaminating the zoo environment (Dubey, Gendron-Fitzpatrick et al., 1988; Basso et

al., 2005). Unlike the domestic cat, Pallas cats with high titers before pregnancy can give birth to infected kittens (Basso et al., 2005). In the present study the seropositive Pallas cat had a MAT titer of 1:3,200 or higher.

Serological prevalence in subhuman primates

New World monkeys and lemurs are highly susceptible to clinical toxoplasmosis (Dubey and Beattie, 1988). Four of the 6 ring-tailed lemurs (*Lemur catta*) housed together in the Cincinnati Zoo (one of the participant zoos in the present study) died of acute toxoplasmosis (Dubey et al., 1985). Antibodies to *T. gondii* were not found in the 2 unaffected lemurs 3 wk after the death of the cagemates, indicating that there were no infected survivors (Dubey et al., 1985). In this respect, it is of interest that *T. gondii* antibodies were found in 6 of 37 prosimians in the present study (Table I). Two healthy black- and white-ruffed lemurs (*Varecia variegata variegata*) at zoo A had high MAT titers of $\geq 1:3,200$. Four lemurs from zoo D that had tested negative in 2003 seroconverted by November 2004. From this group, 2 red-ruffed lemurs (*Varecia variegata rubra*) and 1 black lemur (*Eulemur macaco flavifrons*) had titers of $\geq 1:3,200$. The fourth animal was a ring-tailed lemur (*Lemur catta*) with a titer of 1:100. The red-ruffed lemurs were asymptomatic, and the black lemur was affected with an acute pneumonia. There was no evidence of toxoplasmosis at necropsy, and histopathology and culture from lungs demonstrated *Klebsiella pneumoniae*. Spencer et al. (2004) reported acute toxoplasmosis in a captive *Lemur catta*; the diagnosis was verified by isolation of viable *T. gondii* from the tissues of the dead monkey.

In our study 56.7% of the lemurs evaluated were ring tailed, with only 1 individual having a MAT titer of 1:100; toxoplasmosis was confirmed at necropsy by immunohistochemistry and histopathology in this animal. Recently, Yabsley et al. (2007) reported *T. gondii* antibodies in 3 of 52 from free-ranging ring-tailed lemurs from St. Catherine's Island, Georgia; these animals had MAT titers of 1:50.

Antibodies to *T. gondii* were not found in any of the 36 New World monkeys tested. Most of these monkeys were kept in closed exhibits inside buildings, thus access from feral cats is less likely. Another explanation is that these animals are highly susceptible to toxoplasmosis, and all infected animals could have died of toxoplasmosis.

Serological survey in avian species

Initially we wanted to test seroprevalence in carnivorous and noncarnivorous birds. Unfortunately, because of the small size of the majority of birds and difficulty in handling them, we could not obtain a representative number of plasma from all the groups. As a result, close to 90% of the birds tested were birds of prey; probably because of their larger size, extra plasma was collected and stored. In our study 2 captive eagles tested positive to *T. gondii* antibodies with a low titer of 1:25. These animals are kept in closed exhibits, protected by a wire mesh; however, small mammals are able to enter the exhibit and may be consumed by the predatory birds. Clinical toxoplasmosis in birds of prey is rare (Dubey, 2002).

Feral cats

Feral cats have been incriminated as probable sources of *T. gondii* infections and outbreaks in captive zoo animals in the United States (Riemann et al., 1974; Boorman et al., 1977; Jensen et al., 1985; Gorman et al., 1986; Patton et al., 1986; Dubey, Ott-Joslin et al., 1988; Stover et al., 1990; Junge et al., 1992; Pertz et al., 1997), but to our knowledge seroprevalence in feral cats in the zoos has not been documented (Spencer et al., 2003). The 29.4% seroprevalence found in feral zoo cats is not different from the prevalence of feral cats in the United States (reviewed in Conrad et al., 2005), including feral cats from Ohio, Iowa, and Illinois (Smith et al., 1992; Dubey et al., 1995, 2002). Ideally, we wanted to test a larger sample of feral cats but could not obtain permission to trap cats in some zoos.

Detection of *T. gondii* oocysts

Toxoplasma gondii oocysts were not found in any of the samples tested from the captive wild felines and feral cats. These results were expected, since epidemiological studies have shown that in the United States less than 1% of cats are shedding *T. gondii* oocysts at any given moment; consequently it is difficult to detect shedding in any given cat by coprological methods (Dubey, 2004). Also, all of the wild felids that were sampled for *T. gondii* oocysts were adult animals, and 42% were seropositive for *T. gondii* antibodies, so one would expect that oocysts shedding had already occurred (Dubey and Frenkel, 1972).

Epidemiological survey

Although sources of infection could not be determined, the finding of 63.3% seropositivity in marsupials suggests that captive animals are primarily being exposed to the parasite through ingestion of oocysts, either eliminated by feral cats or by mechanical transportation of oocysts shed by captive wild felids and passed to the susceptible zoo species through the keeper's clothing, boots, or cleaning equipment, or by birds and insects serving as transport hosts. Feral cats have been incriminated as probable sources of *T. gondii* infections and outbreaks in captive zoo animals.

Consumption of unwashed raw vegetables and fruits is indicated as a risk factor for acquiring *T. gondii* in humans. In our study analysis by logistic regression showed that zoos that wash fruits and vegetables had a reduced risk for *T. gondii* infection, but the result was not statistically significant (OR = 0.9, $P = 0.75$). Only 3 of 8 zoos reported washing vegetables before offering them to the animals; therefore, this could be a source of infection for herbivores. *Toxoplasma gondii* oocysts are highly resistant, and there is no easy and convenient method to kill oocysts on fruits and vegetables, other than cooking until the temperature reaches 60 C (Dubey, 1998). Although irradiation, ultraviolet, and high-pressure treatments can kill *T. gondii* oocysts, such methods have not become routine (Dubey et al., 1998; Lindsay et al., 2005; Wainwright et al., 2007).

Toxoplasma gondii epizootics reported worldwide have implicated ingestion of *T. gondii* tissue cysts as the most probable source of infection in their animal collection. In our study all of the participating zoos fed their carnivores almost exclusively commercial frozen meat products (Nebraska brand frozen meat

diet, Central Nebraska Packing, Inc., North Platte, Nebraska, and Dallas Crown, Inc., Kaufman, Texas), supplemented 1 or 2 times a week with shank or knuckle bones. Freezing meat overnight in a household freezer is an efficient means of killing most of the *T. gondii* tissue cysts (Dubey, 1974). However, the temperature in the freezer and the thickness of the meat samples are important. Under controlled conditions, tissue cysts were rendered noninfective when internal temperature of the meat reached -13°C (Kotula et al., 1991). Nevertheless, it must be noted that holding temperatures in freezers vary from institutions, and some of them reported temperatures of -2°C . All of our zoos also reported seeing birds and small mammals inside the carnivorous exhibits, and this could also serve as a source of infection.

The fact that we found exhibiting animals in open enclosures had such a significant risk factor (OR 3.22, $P = 0.00$) strengthens the assumption that captive animals housed in our participating zoos are predominantly being exposed through ingestion of oocysts. In all of the zoos visited, macropods were held in open exhibits, in contrast to the other groups that were generally housed in closed exhibits. Large ground-dwelling cats are generally in open areas, but medium and small cats that are able to climb trees are housed in closed exhibits.

ACKNOWLEDGMENTS

The authors thank O. C. H. Kwok for technical assistance and acknowledge the assistance of authorities at Toledo Zoo, Columbus Zoo and Aquarium, Cleveland Metroparks Zoo, Cincinnati Zoo and Botanical Garden, Indianapolis Zoo, Milwaukee County Zoo, St. Louis Zoo, and Louisville Zoo for their participation and collaboration in this study.

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